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In situ analysis of transforming growth factor-βs (TGF-β1, TGF-β2, TGF-β3), and TGF-β type II receptor expression in malignant melanoma

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We have analysed, by *in situ* hybridization, mRNA expression of TGF-β1, TGF-β2, TGF-β3, and of TGF-β type II receptor in benign melanocytic naevi, primary melanomas, and in skin metastases of malignant melanomas. Our results show that melanoma progression correlates with overexpression of TGF-β. All skin metastases and most primary melanomas invasive to Clark's level IV–V revealed specific TGF-β2 mRNA and protein expression. However, expression of this cytokine was not observed in benign melanocytic lesions and was detected only in one of five early primary melanomas investigated. Some primary melanomas and skin metastases also revealed specific TGF-β1 mRNA signals although expression of this isoform was not found in benign naevi. TGF-β3 expression, which was only barely detectable in benign melanocytic lesions, was enhanced in some skin metastases. Interestingly, the epidermis overlaying melanomas revealed lower levels of TGF-β3 mRNA expression than epidermis of healthy skin or epidermis adjacent to benign naevi, thereby suggesting that paracrine mechanisms between tumour cells and keratinocytes may influence melanoma development. In primary melanomas TGF-β type II receptor mRNA signals were much more heterogeneously distributed when compared to benign melanocytic naevi, suggesting variable degrees of TGF-β resistance among melanoma cells within individual lesions. However, melanoma progression appeared not to be correlated with a complete loss of TGF-β type II receptor gene expression, since all skin metastases revealed clearly detectable although heterogeneous levels of TGF-β type II receptor mRNA expression.

Introducton

Transforming growth factor-beta (TGF-β) is the name given to a family of structurally and functionally related cytokines which consist of three highly homologous mammalian isoforms, designated TGF-β1, TGF-β2, and TGF-β3. Although expression of TGF-β isoforms is differentially regulated, these pleiotrophic peptides show similar biological effects in most experimental systems. TGF-βs control cell proliferation and differentiation processes, promote cell motility and angiogenesis, have potent immunosuppressive effects, stimulate synthesis of specific matrix proteins and integrins, and are mediators of epithelial-mesenchymal interactions during embryogenesis and wound healing (reviewed in 1,2). TGF-βs exert their effects via heteromeric complexes of TGF-β type I (TβR I) and type II receptors (TβR II). A recent study has shown that binding of TGF-β to TβR II is required for the recruitment and activation of TβR I (3). Since TβR II can interact with several type I receptors (4,5,6), the specificity of the biological response to ligand appears to be defined by the particular type I receptor, thus providing a rationale for the multifunctional nature of TGF-βs.

Recently, it has been suggested that escape from paracrine or autocrine growth control by TGF-β during carcinogenesis could involve genetic changes in the TβR II gene itself or altered expression of its mRNA (7). Moreover, TGF-β switches from an inhibitor of tumour cell growth to a stimulator of growth and invasion during human colon carcinoma progression (8). Interestingly, elevated TGF-β mRNA levels have been described in cell lines derived from diverse malignancies (9), and increased expression of TGF-β *in situ* has been observed in glioblastomas (10) and breast carcinomas (11). An involvement of TGF-β in tumour progression has been further substantiated by the finding that highly immunogenic fibrosarcoma cells transfected with TGF-β1 escaped immunosurveillance (12). Moreover, TGF-β2 has been shown to suppress T-cell mediated immunity associated with glioblastoma multiforme (13), and anti-TGF-β antibodies inhibited breast cancer cell tumorigenicity and increased mouse spleen natural killer cell activity (14).

In vitro studies have shown that normal melanocytes and some melanoma cells express TGF-β (15). However, normal melanocytes are growth inhibited by TGF-β, whereas melanoma cells show various degrees of TGF-β resistance (16,17). To test the hypothesis, that an altered TGF-β expression or response may be implicated in melanoma progression *in vivo*, we have investigated, by *in situ* hybridization, expression of the three TGF-β isoforms and of TβR II, and by immunohistochemistry, the protein distribution of TGF-β2, in histological sections of benign melanocytic naevi, primary melanomas, and skin metastases of malignant melanoma.

Materials and methods

Preparation of [^{35}S]-labeled riboprobes

All riboprobe templates used in this study were described previously (18). 'Sense' and 'antisense' RNA probes were labeled according to the instructions of the manufacturer (Boehringer Mannheim: RNA Transkription Kit; Kat.Nr. 999644) with α-^{35}S-UTP (>400 Ci/mmol, Amersham, Nr. SJ 263) to a specific activity of $>10^9$ dpm/μg using SP6, T3 or T7 RNA polymerase. Labeled riboprobes were extracted with phenol/chloroform and free nucleotides were removed by using a Sephadex G50 columne. RNA was precipitated in 1 vol. 7 M ammonium acetate/3.5 vol. ethanol overnight at –20°C, and then resuspended to an approximate concentration of 250 000 cpm/μl (5× concentrated stock solution) in 50% deionized formamide containing 20 mM dithiothreithol and stored at –70°C.

In situ hybridization

Biopsies were fixed overnight at 4°C in a freshly prepared solution of 4% paraformaldehyde in phosphate buffered salt solution (PBS), and then embedded in paraffin. 8 μm paraffin sections were placed on 3-aminopropyltriethoxysilane treated slides, which bind sections covalently on the glass surface and prevent loss of sections during the experimental procedures. Paraffin sections were deparaffinized in xylene and absolute ethanol and air dried. Following rehydration with decreasing ethanol solutions sections were postfixed with 4% paraformaldehyde in PBS for 5 min, rinsed in PBS and

H_2O, depurinated for 20 min with 0.2 N HCl at room temperature, treated for 30 min with 2×SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0) at 70°C, dehydrated with increasing ethanol solutions and finally air tried. Prehybridization was performed at 54°C for 3 h in 50% deionized formamide, 10% dextransulfate, 0.3 M NaCl, 10 mM Tris, 10 mM sodium phosphate pH 6.8, 20 mM dithiothreitol, 0.2×Denhardt's reagent, 0.1 mg/ml *E.coli* RNA, and 'cold' 0.5 µM α-S-UTP. Hybridization was carried out overnight in the same mix supplemented with 5×10^4 cpm/µl α-^{35}S-UTP labelled RNA probe in a humified chamber at 54°C. Slides were washed in hybridization solution without dextransulfate, RNA, and 'cold' UTP containing 50% deionized formamide and 10 mM dithiothreitol at 55°C two times for 1 h and equilibrated for 15 min in a buffer solution consisting of 0.5 M NaCl, 10 mM Tris, 1 mM EDTA, 10 mM DTT pH 7.5. Sections were then treated with 50 µg ml^{-1} RNase A in equilibration buffer for 30 min at 37°C to remove non-specific bound probe. Sections were washed in 2×SSC for 1 h and then in 0.1×SSC for 1 h at 37°C. Slides were then sequentially dehydrated in 65%, 85% and 95% (v/v) ethanol solutions containing 300 mM ammonium acetate and in absolute ethanol before being air dried. Sections were coated with a 1:2 dilution of Ilford K5 photoemulsion, air dried, and exposed for 12 days in a light safe box containing silica gel at 4°C. Slides were developed in D19 developer (Kodak), fixed in 30% sodium thiosulfate and stained with haematoxylin and eosin. The pattern of hybridization signals on autoradiographed sections was analysed using a photomicroscope and brightfield/darkfield illuminations. For a semiquantitative analysis of mRNA expression, the average number of silver grains per cell was calculated within individual tissues. mRNA expression was defined as 'not detectable' if the average number of silver grains was less than two per cell and therefore not clearly distinguishable from non-specific background.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections using a rabbit polyclonal IgG, which recognizes residues 352–377 of human TGF-β2 (Santa Cruz Biotech). Sections were de-waxed in xylene, rehydrated in decreasing ethanol solutions, and incubated for 1 h in PBS containing 1% BSA. The primary antibody was diluted (1 µg/ml) in PBS containing 0.2% BSA and applied overnight at 4°C. Antibody staining was performed using the StrAviGen Super Sensitive detection kit using fast red as substrate (BioGenex, San Ramon, CA). Sections were counterstained with haematoxylin.

Results

Specificity of signals

The specificities of the TGF-β1/2/3 and TβR II probes were determined by Northern blot analysis. By using riboprobes encoding the three mature TGF-β isoforms, we have previously shown that no cross-hybridization occurs during *in situ* hybridization (18). The specificity of the hybridization was further controlled by using the corresponding 'sense' riboprobes as negative controls which gave very weak and uniformly distributed non-specific background signals comparable to the non-specific signals shown in Figures 1a, 1b and 2b.

Specificity of immunostaining was controlled by using non-immune serum which revealed no specific tissue staining (not shown).

Benign melanocytic naevi

Four benign melanocytic naevi (1 compound, 1 junctional, 2 dermal) were investigated (Table I). In benign melanocytic naevi, specific expression of TGF-β1 and TGF-β2 mRNA was not detectable (Figures 1a,b). Only weak TGF-β3 hybridization signals (Figure 1c) were observed in three benign melanocytic lesions, although the epidermis (Figure 1c) and hair follicle epithelia (not shown) revealed abundant TGF-β3 mRNA expression. Expression of TβR II mRNA (Figure 1d) was clearly detectable in all four naevi, the level of expression being comparable to that observed in epidermal keratinocytes (Figure 1d) and vascular endothelia (not shown).

Primary melanomas

Five early melanomas according to Clark's level II–III, and five advanced melanomas according to Clark's level IV–V were investigated (Table I). The majority of early primary melanomas revealed no or only barely detectable expression of TGF-β1, TGF-β2 and TGF-β3 mRNA (Figure 2a–c). Abundant TGF-β1 mRNA expression was detected in two early melanomas and in one advanced melanoma. Specific signals for TGF-β2 mRNA expression were found only in one early melanoma, although three advanced melanomas revealed abundant TGF-β2 expression. Two early melanomas and two advanced melanomas revealed levels of TGF-β3 mRNA expression which were stronger than those observed in benign naevi. Interestingly, the epidermis overlaying most primary melanomas (Figure 2c) revealed significantly lower levels of TGF-β3 mRNA expression (Figure 2c) when compared with epidermis of healthy skin (not shown) or epidermis overlaying

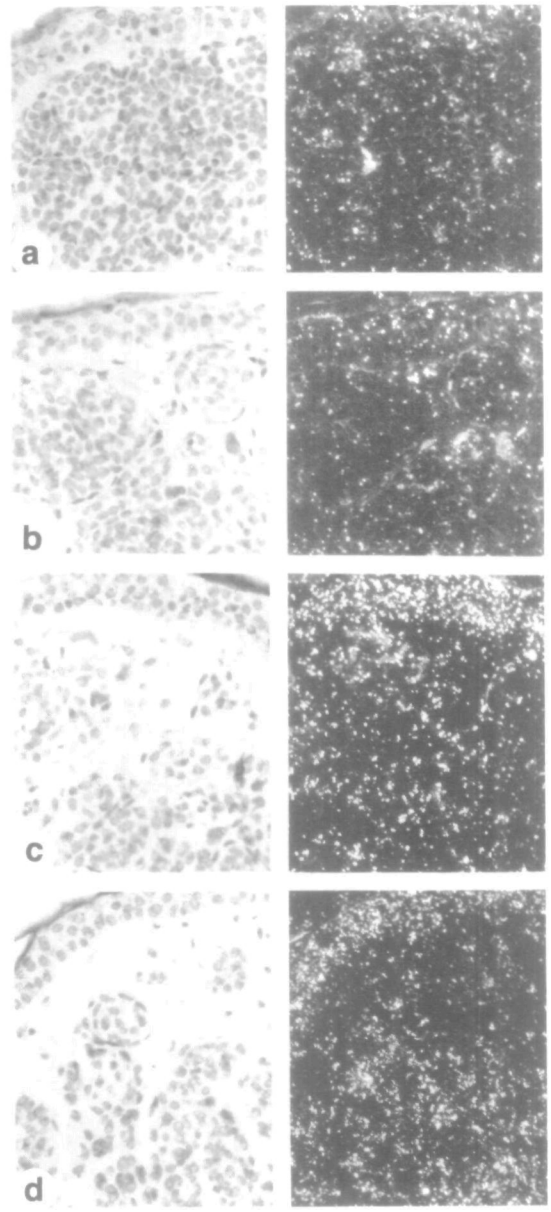

Fig. 1. *In situ* hybridization analysis of (**a**) TGF-β1, (**b**) TGF-β2, (**c**) TGF-β3, and (**d**) TβR II mRNA expression in naevus naevocellularis (left) Brightfield illuminations, (right) darkfield illuminations. (a) No specific TGF-β1 hybridization signals are visible. (b) Expression of TGF-β2 mRNA is not visible. (c) Naevus cells shows only barely detectable levels of TGF-β3 mRNA expression, although expression of TGF-β3 mRNA is clearly visible in the epidermis. (d) Abundant TβR II mRNA signals are visible both in naevus cells and in the epidermis.

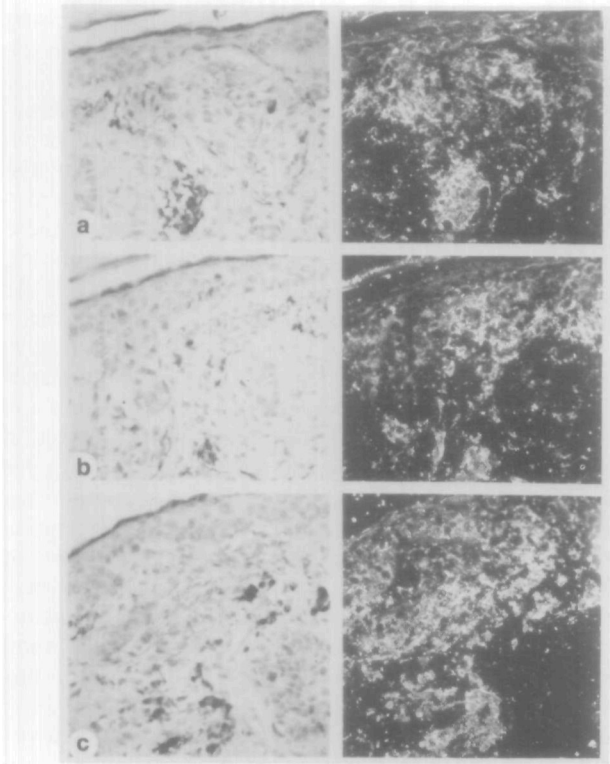

Fig. 2. *In situ* hybridization analysis of (**a**) TGF-β1, (**b**) TGF-β2, and (**c**) TGF-β3 mRNA expression in an early primary melanoma. (left) Brightfield illuminations; (right) darkfield illuminations. (a) TGF-β1 hybridization signals are only barely discernible within the tumour. (b) No specific TGF-β2 hybridization signals are visible. (c) TGF-β3 hybridization signals are only barely detectable both in the epidermis and within the tumour. Note, the bright spots in darkfield illuminations do not represent hybridization signals but light reflections from pigment granula.

Table I. Traceability of TGF-β1, TGF-β2, and TGF-β3 mRNA expression in melanocytic lesions by *in situ* hybridization

	TGF-β1	TGF-β2	TGF-β3
Benign naevi (n = 4)	(0/4)	(0/4)	(3/4)
Early melanomas (n = 5)	(2/5)	(1/5)	(2/5)
Advanced melanomas (n = 5)	(1/5)	(3/5)	(2/5)
Skin metastases (n = 8)	(3/8)	(8/8)	(4/8)

benign naevi (Figure 1c). Expression of TβR II was clearly detectable in all primary melanomas. However, in primary melanomas TβR II hybridization signals (Figure 3) were much more heterogeneously distributed when compared with benign naevi (Figure 1d).

Skin metastases

Eight skin metastases from malignant melanomas were investigated. All skin metastases (Table II) revealed clearly detectable but various levels of TGF-β2 mRNA (Figure 4b) and protein (Figure 5). Three metastases (Table II) showed also specific TGF-β1 hybridization signals (Figure 4a), and two (Table II) revealed levels of TGF-β3 mRNA expression (Figure 4c) which were markedly stronger than those observed in some benign naevi and primary melanomas (Figures 1c, 2c). The level of TβR II mRNA expression varied among individual metastases (Table II) and was stronger in endothelial cells than in melanoma cells (Figure 4d).

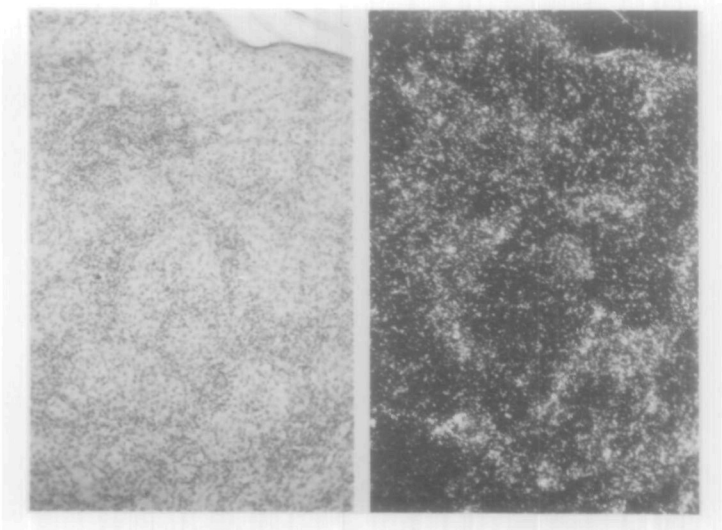

Fig. 3. *In situ* hybridization analysis of TβR II mRNA in an advanced primary melanoma. (left) Brightfield illumination; (right) darkfield illumination. TβR II hybridization signals are very heterogeneously distributed within the tumour.

Table II. Semiquantitative analysis of TGF-β1, TGF-β2, TGF-β3, and TβR II mRNA expression in skin metastases of malignant melanomas

Biopsy no.	TGF-β1	TGF-β2	TGF-β3	TβRII
93.1983	–	++	–	+
93.2702	+++	+++	+	++
93.3766	+	+	–	+++
93.3832	–	++	+	+
93.737	–	++	–	++
94.491	++	+	++	+
94.617	–	++	++	+++
94.4092	–	++	–	+

Average number of silver grains per cell: – = < 2; + = 2–5; ++ = 6–9; +++ = > 9.

Discussion

A previous study has shown that TGF-β3 is the predominantly expressed isoform in human epidermis (18). Since TGF-β is a potent inhibitor of melanocytic proliferation *in vitro* (16,17), epidermal TGF-β3 may have a physiological function to control cell division and/or differentiation of melanocytes. The finding that TGF-β3 mRNA expression is decreased in the epidermis overlaying primary melanomas, when compared to normal epidermis, suggests that melanoma cells may suppress keratinocytic TGF-β3 expression via a paracrine mechanism. Conversely, it is tempting to speculate that lack of TGF-β3 expression in the epidermis could precede melanoma development and promote clonal expansion of transformed melanocytes. Melanomas may therefore arise predominantly at sites of low epidermal TGF-β3 expression.

Furthermore, the heterogeneous pattern of TβR II expression in primary melanomas may indicate variable degrees of TGF-β resistance among melanoma cells, a finding which might explain the decreased growth-inhibitory effect of TGF-β on melanoma cells *in vitro* (16,17). However, melanoma progression does not imply a complete loss of TβR II mRNA expression since all metastases revealed clearly detectable but various levels of TβR II expression. Other mechanisms may therefore contribute to the altered TGF-β reponsiveness of

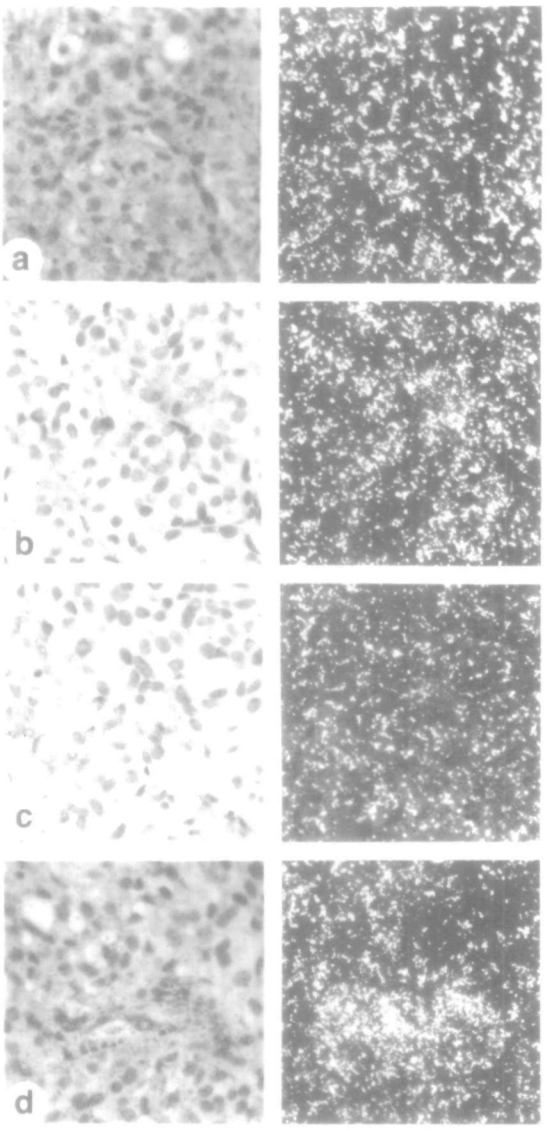

Fig. 4. *In situ* hybridization analysis of (**a**) TGF-β1, (**b**) TGF-β2, (**c**) TGF-β3, and (**d**) TβR II mRNA expression in a skin metastasis (biopsy no. 93.2702) of malignant melanoma (left) Brightfield illuminations, (right) darkfield illuminations. Abundant (a) TGF-β1 and (b) TGF-β2, and weak (c) TGF-β3 expression is visible in melanoma cells. (d) Expression of TβR II is stronger in endothelial cells than in melanoma cells.

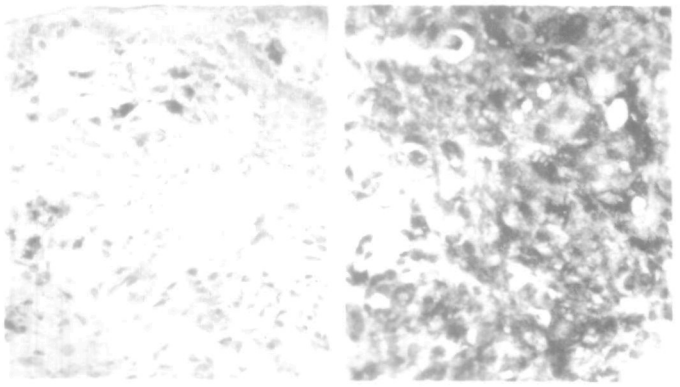

Fig. 5. Immunohistochemical analysis of TGF-β2 protein distribution in malignant melanoma. (left) Early primary melanoma. No specific immunostaining is visible. (right) Skin metastasis of malignant melanoma. TGF-β2 immunoreactivity is clearly visible throughout the tumour.

melanoma cells, such as TβR II gene mutations or processing defects, missing TGF-β type I receptors, or alterations in the intracellular TGF-β signal transduction pathways.

The present study confirms the recently puplished observation that TGF-β2 in malignant melanoma correlates with depth of tumour invasion (19). In addition, we have shown that overexpression of TGF-β1 and/or TGF-β3 mRNA may also occur in metastatic melanoma. However, a correlation of these isoforms with melanoma progression appears less significant, since not all metastases revealed elevated levels of TGF-β1 and/or TGF-β3 transcripts. Expression of TGF-β1 has been found in cultured melanocytes (15) although we failed to detect specific TGF-β1 mRNA expression in benign melanocytic lesions, suggesting that the level of TGF-β1 expression in benign melanocytic lesions is below the limit of detection by *in situ* hybridization. However, the growth medium for melanocytes is supplemented with growth factors and phorbol ester, the latter being a potent inducer of TGF-β1 transcription (20,21). It remains therefore questionable if production of TGF-β1 by melanocytes *in vitro* has a physiological relevance. A role of TGF-β3 to control melanocytic growth or differentiation appears more likely, since normal epidermis and several benign naevi showed detectable mRNA expression of this isoform.

It appears that in melanomas a selective pressure favors overproduction of TGF-β which might promote tumour progression by diverse possible mechanisms. TGF-β has potent immunosuppressive effects, because it inhibits IL-1 stimulated proliferation of T-lymphocytes (22), suppresses cytokine stimulated activation of natural killer cells (23), and appears to be an important negative regulator of MHC class I and class II expression (24). A recent review of the literature (25) has shown that HLA class I antigens are not detectable in about 40% of metastatic melanoma lesions. Conversely, the cytotoxicity of the $V\beta16^+$ T cell line, which has been generated by *in vitro* expansion of tumour infiltrating lymphocytes of a patient with regressing melanoma (26), is HLA class I restricted. Therefore, secretion of bioactive TGF-β2 by melanoma cells may well contribute to suppress local immunosurveillance, although this mechanism appears not to be the only way which allows melanoma cells to escape immune recognition, since Huber *et al.* (27) have shown that some melanoma cell lines can inhibit, by direct contact, the proliferation of tumour infiltrating lymphocytes via a TGF-β2 independent pathway. The abilities of TGF-βs to induce angiogenesis and to promote the formation of stroma (28) may be another role of these polypeptides in the maintenance and progression of transformed cells in the host (29). Interestingly, human melanoma cells have been shown to require ligation of the integrin $a_v\beta_3$ to sustain viability and growth in three dimensional dermal collagen (30). Since TGF-β up-regulates expression of integrin $a_v\beta_3$ (31), overexpression of TGF-β may contribute to rescue melanoma cells from apoptosis. Another possible pathomechanism may implicate the metastasis suppressor gene nm23 (32). In well-differentiated human colon carcinoma cells nm23 functions in the TGF-β signalling pathway leading to growth arrest and differentiation, whereas colon carcinoma cells which have progressed to a more aggressive phenotype have lost nm23 function and use TGF-β to stimulate growth and invasion (33). Since expression of the nm23 gene has also been inversely correlated with tumour metastatic potential in human malignant melanomas (34) a similar mechanism may contribute to melanoma metastasis.

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